Supporting Information

Blurton-Jones et al. 10.1073/pnas.0901402106

SI Methods

Mice. All animal procedures and use were performed in strict accordance with National Institutes of Health and University of California guidelines. 3xTg-AD mice have previously been characterized (4, 5). The mice were generated by comicroinjecting transgenes expressing human APP with the Swedish mutation (KM670/671NL) and human tau with the P301L mutation into single-cell embryos of homozygous $PS1_{M146V}$ knock-in mice. Homozygous 3xTg-AD used in this study are maintained on a hybrid C57BL6/129 background by performing homozygous × homozygous crosses from within an extensive colony. Nontransgenic control mice are maintained in a similar fashion by crossing wild-type hybrid C57BL6/129 mice with each other. All mice are housed on a 12 h light/dark schedule with ad libitum access to food and water. Both C57Bl6 and 129 background strains have the identical MHC haplotype (H-2b) as the C57Bl6-derived NSC used in this study.

Stereotactic Surgeries. Bilateral intrahippocampal injections of GFP-NSCs or vehicle were performed using a stereotaxic apparatus and the following coordinates relative to Bregma: AP: −2.06, ML: ±1.75, DV: −1.75. Injections were targeted to the hippocampal fissure, a cell sparse region located just dorsal to molecular layer of the dentate gyrus. Mice were anesthetized with isofluorane, placed in the stereotax and injected with either 100,000 NSCs per side (2 μl/injection) or vehicle (1× HBSS with 20 ng/ml hEGF) as a control treatment using a 5 μl Hamilton microsyringe (33-gauge) and an injection rate of 0.5 μl/min. For BDNF injections the same coordinates and methods were used to deliver 0.25 μg recombinant BDNF in 2 μl (PeproTech) or vehicle (HBSS). Accurate placement of the injection to the targeted region was confirmed for all animals by visualization of the needle tract within coronal brain sections.

Behavioral Analysis. Hippocampal-dependent learning and memory was examined by a blinded observer, using Morris water maze and novel object recognition tasks following standard protocols (4). For MWM, animals were habituated to a 1 m diameter circular pool filled with opaque water maintained at 29 °C. During training, mice were paced into the pool and allowed to find and climb onto a submerged 12 × 12 cm platform for 4 trials per day. After 6 days of training, the escape platform was removed and mice were tested 24 h later to assess retention of spatial memory. For probe trials, the latency to reach the former location of the platform was measured and the number of times the mouse crosses the former platform location recorded. Probe trial latencies are consistently shorter than the last day of training as the mouse has an additional day of consolidation and does not need to climb onto a platform to successfully complete the task. Training and probe trial data were analyzed by multifactor ANOVA with Fischer's probable least-squares difference posthoc test to determine significant differences ($P < 0.05$). For context-dependent novel object, mice were exposed to 2 identical objects (i.e., 2 toy balls) in a round cage for 5 min and then 2 other identical objects (i.e., 2 small cubes) in a rectangular cage for 5 min. After 24 h, mice were placed into either the round or the rectangular cage in which one of the objects was novel for that context (i.e., a toy ball and a small cube are placed into the round cage). The proportion of time spent investigating the novel "out of context" object versus the in context object was calculated as a recognition index = $[t_{novel}/(t_{novel} + t_{sample})] \times 100$ by a blinded scorer. Recognition indexes were then compared by ANOVA and Fisher's PLSD posthoc tests.

Biochemical Analyses. Five weeks after transplantation, mice were killed by Nembutal overdose and cardiac perfusion with 0.01M PBS (PBS). One side of the brain was frozen on dry ice for subsequent biochemical analysis. The other side was postfixed in 4% paraformaldehyde, and cut on a Vibratome (50 μm, coronal) for immunofluorescent analyses. Half brains (excluding the cerebellum) were processed to isolate soluble and insoluble protein from each animal following standard protocols (3). Briefly, brains were homogenized on ice in 200 mg/ml TPER (Pierce) with protease and phosphatase inhibitor cocktails (Sigma). The homogenate was spun at 100,000 *g* for 1 h at 4 °C in a T865 Sorvall rotor in a L8–70 ultra-centrifuge (Beckman). The supernatant (soluble fraction) was collected and stored at −80 °C. The pellet was resuspended in 70% formic acid and spun as in the previous step. The supernatant was collected and stored at −80 °C. Soluble and insoluble SDS-page Western blots and Aβ sandwich ELISAs were performed following standard protocols as previously described (13). BDNF ELISA was performed following the manufacturers protocol (Promega).

Immunofluorescent Labeling and Primary Antibodies. Fluorescent labeling followed previously described protocols (26). Briefly, coronal brain sections were rinsed 3 times then placed in blocking solution (PBS + 0.02% Tx-100, 5% goat serum) for 1 h. Primary antibodies were diluted in fresh blocking solution and applied overnight at 4 °C. Sections were rinsed 3 times in PBS and then incubated for 1 h in appropriate Alexa Fluor secondary antibodies. Following 3 additional rinses, sections were mounted on slides and coverslipped using Fluoromount G (Southern Biotechnology). Specificity of primary antibodies was confirmed by Western blot or conformation-sensitive ELISA (A11 and OC), and primary antibody omission controls. The following primary antibodies were used: 6E10 (Signet), oligomeric Aβ and fibrillar Aβ (A11 and OC respectively, gift of C. Glabe, University of California, Irvine), HT7 (Innogenetics), phospho-Tau (Ser 199/202; Invitrogen), PHF-1 (gift of P. Davies, Albert Einstein College of Medicine, Bronx, NY), synaptophysin, β-actin (Sigma), BDNF and Doublecortin (Santa Cruz Biotechnology), Pro-BDNF (gift of D. Cribbs, University of California, Irvine), GFAP (Dako), β-III-tubulin, GFP, GalC and CNPase (Millipore).

Confocal Microscopy and Quantification. Sections were imaged with a Bio-Rad Radiance 2100 confocal system using lambda-strobing mode. Images represent either single confocal Z-slices or Z-stacks. For analysis of differentiation, sections were double-labeled for GFP, Doublecortin, GFAP, or GalC and quantified under epifluorescence by a blinded observer. A total of 2110 GFP-labeled cells were quantified and the proportion of those cells that double-labeled with neuronal or glial markers is provided in Table S1. For synaptic density, Z-stack images (15 slices, 1 μm step size) were captured from coded slides using identical settings. Grayscale Z-stack images were then analyzed with Image J. Three square regions of interest (ROIs) were randomly defined within CA1 stratum radiatum and mean pixel intensity computed. Background intensity was measured within the lateral ventricle and subtracted from ROI measurements. The 3 fields were averaged for each sample and group means statistically compared.

Microfluidic Chambers. Microfluidic chambers were fabricated as previously described to form somal and axonal chambers interconnected by 450 μm long, 10 μm wide, and 3 μm high microchannels (26). E18 primary rat neurons were isolated following standard protocols (26) and plated into the somal compartment. By 7 days in vitro, axons had begun to extend through the channels into the axonal chamber. At this timepoint, cultures ($n = 7$ per condition) were treated with one of 3 treatments: (*i*) control media conditioned by prior exposure to primary neurons, (*ii*) NSC-conditioned media which had been exposed to differentiated NSCs for 5 days, (*iii*) NSC-conditioned media (same media as in *ii*) in which BDNF had been removed by immunoprecipitation. After 3 days of treatment, chambers were fixed, labeled for βIII-tubulin, and axonal outgrowth was quantified by a blinded observer as described in *SI Methods*.

Statistical Analysis. All statistical analysis was performed using Statview 5. Comparisons between multiple groups used analysis of variance (ANOVA) followed by Fischer's PLSD posthoc tests. Comparisons between 2 groups used students T-test. Differences were considered significant when $P < 0.05$ for all tests.

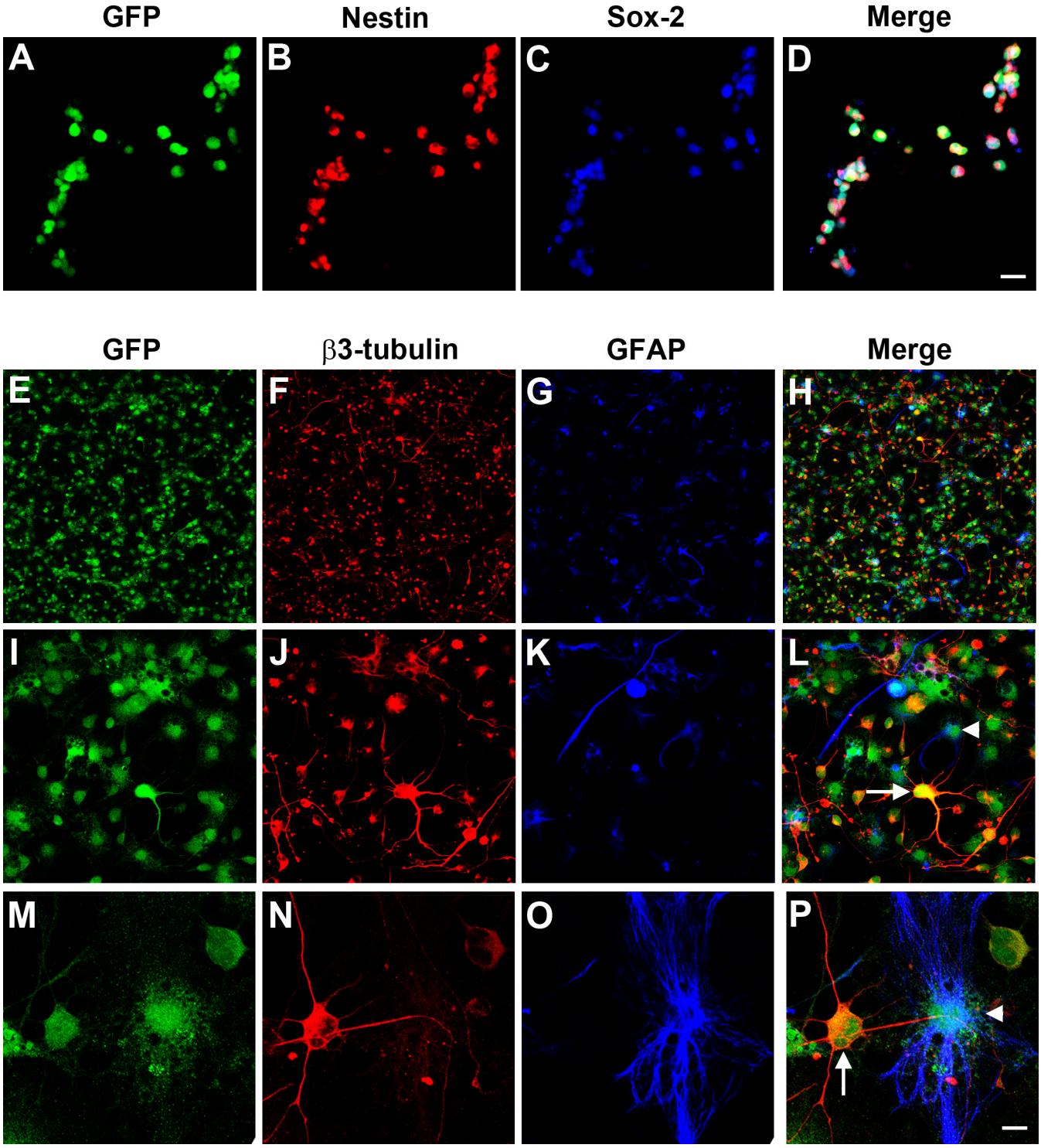

Fig. S1. Cells derived from GFP transgenic mice express neural stem cell markers and are multipotent. To characterize the GFP-NSCs used in this study, adherent cultures were examined by immunocytochemistry for GFP (*A*), the NSC markers, nestin (*B*), and sox-2 (*C*) merged image shown in (*D*) and found to colocalize all 3 markers. Removal of mitogen and supplementation of media with B27 induces differentiation of GFP cells into all 3 lineages. Neuronal (β3-tubulin, red) and astrocytic (GFAP, blue) markers are shown in (*E*–*P*). Arrows in (*L*) and (*P*) denote examples of neuronal differentiation whereas arrowheads denote astrocytes. Differentiation of oligodendroglia from GFP-NSCs was also confirmed by immunoreactivity for CNPase (see Fig. 2). (Scale bars, 22 μm in *A*–*D*, 80 μm in *E*–*H*, 24 μm in *I*–*L*, and 9 μm in *M*–*P*.)

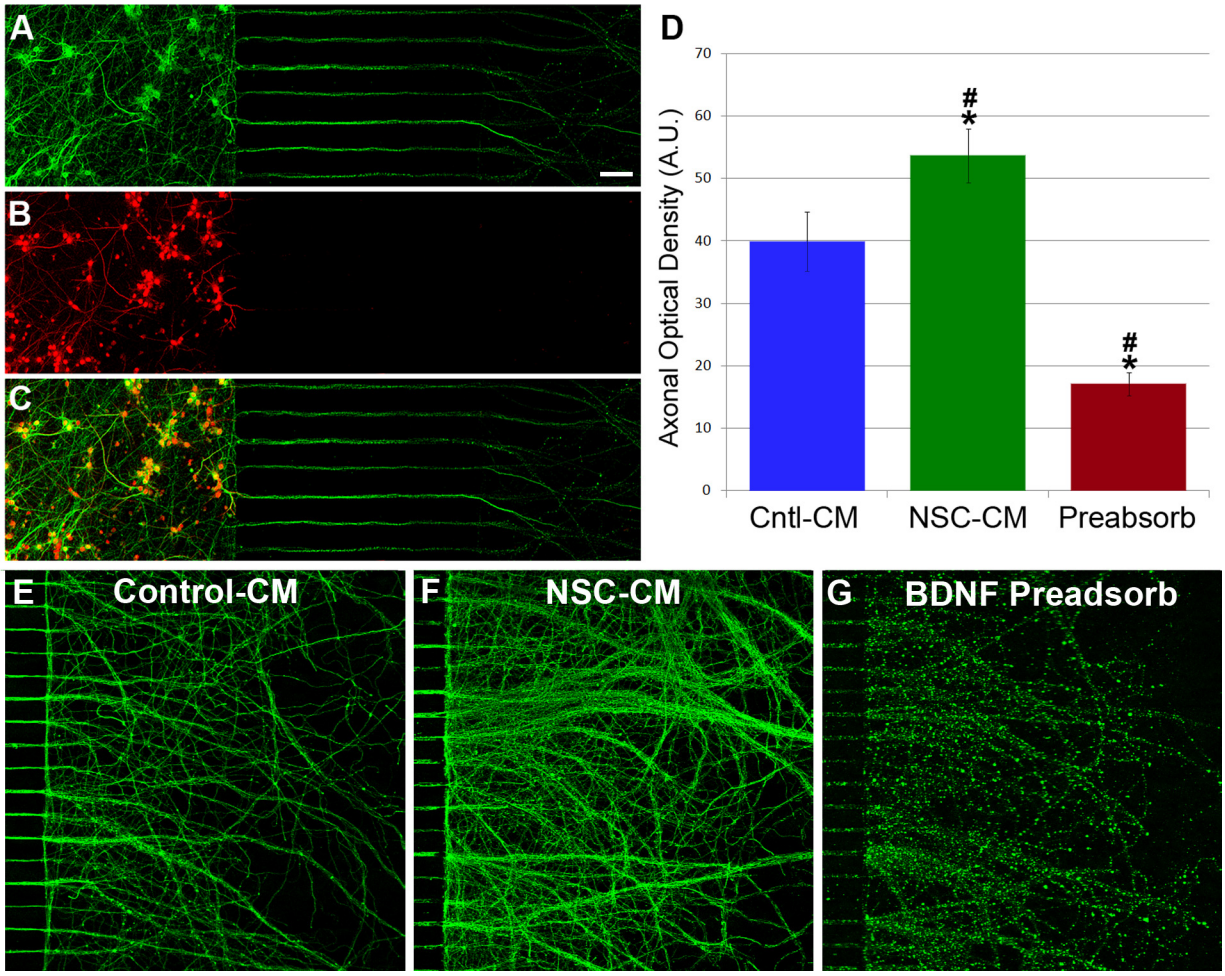

Fig. S2. Microfluidic studies reveal that stem cell-induced neuronal plasticity is mediated via a BDNF-dependent mechanism. To further define the mechanism underlying the NSC-induced neuronal plasticity, we used a novel microfluidic cell culture platform. This device allows primary hippocampal neurons to be cultured in a manner that facilitates quantitative analysis of axonal outgrowth. (*A*) The axonal marker MAP5 (green) demonstrates that axons can extend through channels within the device into a separate chamber. (*B*) In contrast, dendrites (red, MAP2) fail to extend into the second chamber, merged image shown in (*C*). To determine whether NSCs secrete a diffusible factor that enhances neuronal plasticity, primary neuron chamber cultures were treated with control conditioned media (*D* and *E*) versus differentiated NSC-conditioned media (*D* and *F*). Optical densitometry revealed a significant increase in axonal outgrowth and sprouting in response to differentiated NSC conditioned media versus control conditioned media (*D*, ANOVA $P < 0.0001$, Fisher's PLSD $P = 0.0193$). To test whether BDNF mediates this increase, microfluidic chambers were also treated with the same NSC-derived conditioned media in which BDNF had been preabsorbed (*D* and *G*). As demonstrated, removal of BDNF from stem cell conditioned media completely prevented axonal outgrowth even beyond that observed in response to control-conditioned media and even lead to evidence of axonal degeneration (ANOVA $P < 0.0001$, Fisher's PLSD $P < 0.001$), data are presented as mean $\pm$ SEM. Thus, NSC-induced neuronal plasticity appears to be mediated via a BDNF-dependent mechanism. (Scale bars, 60 μm in *A*–*C*, 40 μm in *E*–*G*.)

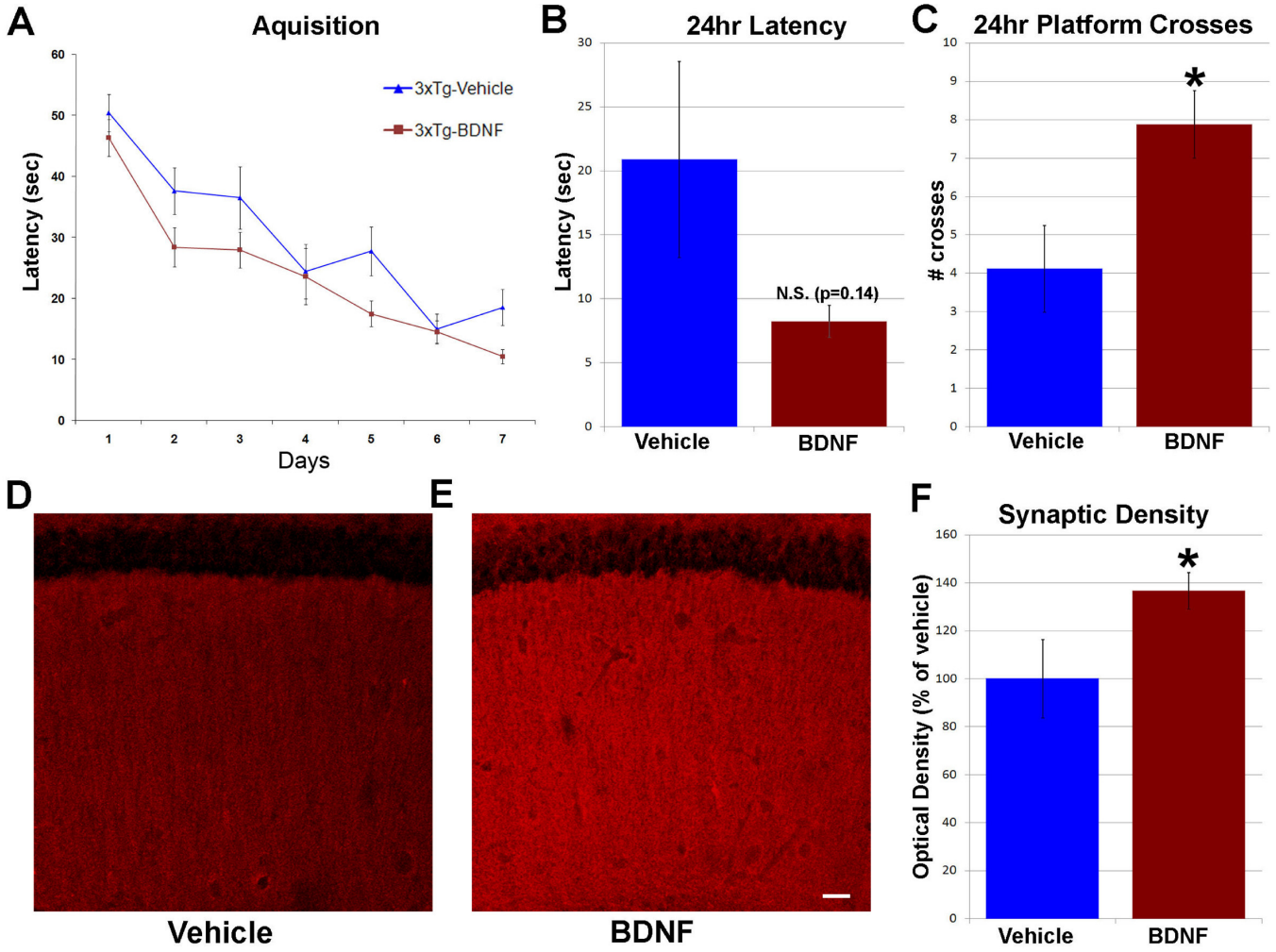

Fig. S3. BDNF mimics the effect of NSCs; improving spatial memory deficits and increasing synaptic density in 3xTg-AD mice. (*A*) During water maze acquisition, BDNF-treated 3xTg-AD mice trended toward shorter escape latencies versus vehicle-treated mice (repeated measures ANOVA $P = 0.06$, Fisher's PLSD, $P = 0.04$ (day 5) and $P = 0.03$ (day 7). (*B*) BDNF-treated animals also exhibited a trend toward shorter latencies during probe trial testing ($P = 0.14$). (*C*) Importantly, BDNF-injected mice exhibited a strong memory for the platforms former location, crossing the area almost twice as often as vehicle-injected controls ($P = 0.02$). Furthermore, confocal optical densitometry revealed that compared to (*D*) vehicle-injected 3xTg-AD mice, (*E*) BDNF-injected mice exhibit significantly more synaptophysin immunoreactivity (F, $P = 0.04$). Thus, intrahippocampal BDNF injection mimics many of the effects of NSC transplantation. Data are presented as mean ± SEM. (Scale bar, 40 μm in *D*–*E*.)

Table S1. NSC differentiation

	Neurons	Astrocytes	Oligodendrocytes
All animals	5.81% ± 2.3	39.43% ± 2.8	26.44% ± 2.0
3xTg-AD	7.20% ± 3.6	37.75% ± 2.3	25.20% ± 2.7
Wild-type	3.00% ± 1.6	45.03% ± 10.4	28.23% ± 3.3